Human cerebrovascular responses to diving are not related to facial cooling

AlSalahi, Sultan E.; Braz, Igor D.; Ahmed, Amar; Junejo, Rehan T.; Fisher, James P.

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Authors: Sultan E Alsalahi
Igor D Braz
Amar Ahmed
Rehan T Junejo
James P Fisher

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Running Title: Cerebral perfusion and trigeminal nerve stimulation

Abstract: Diving evokes a pattern of physiological responses purported to preserve oxygenated blood delivery to vital organs such as the brain. We sought to uncouple the effects of trigeminal nerve stimulation on cerebral blood flow (CBF), from other modifiers associated with the diving response, such as apnoea and changes in arterial carbon dioxide tension. Thirty-seven young healthy individuals participated in separate trials of; Facial cooling (FC, 3 min) and cold pressor test (CPT, 3 min) under poikilocapnic (Protocol 1) and isocapnic conditions (Protocol 2), facial cooling while either performing a breath-hold (FC +BH) or breathing spontaneously for a matched duration (FC -BH) (Protocol 3), and BH during facial cooling (BH +FC) or without facial cooling (BH -FC) (Protocol 4). Under poikilocapnic conditions neither facial cooling nor CPT evoked a change in middle cerebral artery blood flow velocity (MCA V_{mean}; transcranial Doppler) ($P>0.05$ vs. baseline). Under isocapnic conditions, facial cooling did not change

MCA $_{Vmean}$ ($P>0.05$), whereas CPT increased MCA V_{mean} by 13% ($P<0.05$). Facial cooling with a concurrent BH markedly increased MCA V_{mean} (Δ23%) and internal carotid artery blood flow (ICA_Q; duplex Doppler ultrasound) (Δ26%) ($P<0.001$), but no change in MCA V_{mean} and ICA_Q were observed when facial cooling was accompanied by spontaneous breathing ($P>0.05$). Finally, MCA V_{mean} and ICA_Q were similarly increased by BH either with or without facial cooling. These findings suggest that physiological factors associated with BH, and not facial cooling (i.e., trigeminal nerve stimulation) per se, make the predominant contribution to increases in CBF during diving in humans.

New Findings: What is the central question of this study? Does facial cooling mediated stimulation of cutaneous trigeminal afferents associated with the diving response increase cerebral blood flow or are factors associated with breath-holding (e.g., arterial carbon dioxide accumulation, pressor response) more important in humans? What is the main finding and its importance? Physiological factors associated with breath-holding such as arterial carbon dioxide accumulation and the pressor response, but not facial cooling (trigeminal nerve stimulation), make the predominant contribution to diving response mediated increases in cerebral blood flow in humans.

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Human Cerebrovascular Responses to Diving are not Related to Facial Cooling

Authors: Sultan E AlSalahi[1], Igor D Braz[1,2], Amar Ahmed[1], Rehan T Junejo[1] & James P Fisher[3]

Institutions: [1]School of Sport, Exercise and Rehabilitation Sciences, College of Life and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, UK; [2]University Center of Volta Redonda, Volta Redonda, Rio de Janeiro, Brazil; [3]Department of Physiology, Faculty of Medical & Health Sciences, University of Auckland, New Zealand.

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Corresponding author:

James P. Fisher, PhD
Department of Physiology,
Faculty of Medical & Health Sciences,
University of Auckland,
Park Road, Grafton,
Auckland 1142,
New Zealand.
(+649) 3737599 x86320
Email: jp.fisher@auckland.ac.nz

- **What is the central question of this study?**

Does facial cooling mediated stimulation of cutaneous trigeminal afferents associated with the diving response, increase cerebral blood flow or are factors associated with breath-holding (e.g., arterial carbon dioxide accumulation, pressor response) more important in humans?

- **What is the main finding and its importance?**

Physiological factors associated with breath-holding such as arterial carbon dioxide accumulation and the pressor response, but not facial cooling (trigeminal nerve stimulation), make the predominant contribution to diving response mediated increases in cerebral blood flow in humans.

ABSTRACT

Diving evokes a pattern of physiological responses purported to preserve oxygenated blood delivery to vital organs such as the brain. We sought to uncouple the effects of trigeminal nerve stimulation on cerebral blood flow (CBF), from other modifiers associated with the diving response, such as apnoea and changes in arterial carbon dioxide tension. Thirty-seven young healthy individuals participated in separate trials of; Facial cooling (FC, 3 min) and cold pressor test (CPT, 3 min) under poikilocapnic (Protocol 1) and isocapnic conditions (Protocol 2), facial cooling while either performing a breath-hold (FC +BH) or breathing spontaneously for a matched duration (FC -BH) (Protocol 3), and BH during facial cooling (BH +FC) or without facial cooling (BH -FC) (Protocol 4). Under poikilocapnic conditions neither facial cooling nor CPT evoked a change in middle cerebral artery blood flow velocity (MCA V_{mean}; transcranial Doppler) ($P>0.05$ vs. baseline). Under isocapnic conditions, facial cooling did not change MCA V_{mean} ($P>0.05$), whereas CPT increased MCA V_{mean} by 13% ($P<0.05$). Facial cooling with a concurrent BH markedly increased MCA V_{mean} (Δ23%) and internal carotid artery blood flow (ICA_Q; duplex Doppler ultrasound) (Δ26%) ($P<0.001$), but no change in MCA V_{mean} and ICA_Q were observed when facial cooling was accompanied by spontaneous breathing ($P>0.05$). Finally, MCA V_{mean} and ICA_Q were similarly increased by BH either with or without facial cooling. These findings suggest that physiological factors associated with BH, and not facial cooling (i.e., trigeminal nerve stimulation) per se, make the predominant contribution to increases in CBF during diving in humans.

INTRODUCTION

Diving evokes a characteristic pattern of physiological responses when the face is immersed in cold water (Gooden, 1994; Foster & Sheel, 2005). It is in part activated by a stimulation of the trigeminal nerve that innervates the areas around the forehead and cheeks, but also modulated by mechanisms such as apnoea (Gooden, 1994; Lemaitre *et al.*, 2015). Activation of the diving response results in a parasympathetically mediated reduction in heart rate (HR) and an increase in sympathetic nerve activity which causes peripheral vasoconstriction and increases mean arterial blood pressure (MAP) (Fagius & Sundlöf, 1986; Shamsuzzaman *et al.*, 2014; Fisher *et al.*, 2015; Lemaitre *et al.*, 2015; Lapi *et al.*, 2016; Schlader *et al.*, 2016). It is thought that the diving response serves to preserve blood flow and oxygen delivery to the heart and brain in animals (Butler & Jones, 1997). However, whether this is primarily a result of the trigeminal afferent stimulation or mechanisms associated with apnoea remains incompletely understood in humans.

Regulation of cerebral blood flow (CBF) is complex and highly integrated involving multiple mechanisms with the aim of ensuring continuous perfusion of oxygenated blood to the brain (Ainslie & Brassard, 2014). Several mechanisms likely contribute to the regulation of CBF during the diving response, including neurogenic and hemodynamic factors, neurovascular coupling and changes in blood gases (e.g., partial pressure of arterial carbon dioxide (P_aCO_2)) (May & Goadsby, 1999; Phillips *et al.*, 2015). Notably, underwater submersion in rats causes a redistribution of blood away from peripheral regions such the thoraco-abdominal region towards the head and thorax, a response not exhibited in rats swimming without head submersion (Ollenberger *et al.*, 1998). This is indicative of a key role for the trigeminal nerves in the control of cerebral perfusion during diving and may be explained by the release of vasorelaxant mediators from activated trigeminal nerve cell bodies that project bipolar cells that synapse on extra-cerebral vessels (e.g., the middle

cerebral artery; MCA) (May & Goadsby, 1999). In addition, with activation of trigeminal afferents and cutaneous thermoreceptors during facial cooling, regional cortical sites within the central nervous system are activated and increases local metabolism. These are potentially coupled to increases local blood flow via complex series of cellular events, collectively referred to as neurovascular coupling (Phillips *et al.*, 2015). Activation of the sympathetic nervous system along with changes in hemodynamic factors (e.g., blood pressure, cardiac output) can potentially impact CBF during facial cooling (Fisher *et al.*, 2015). Moreover, arterial blood gases concentrations can play a critical part in cerebral blood regulation. When the diving response is associated with apnoea, hypercapnia and hypoxia evoke cerebral vasodilation, and hypercapnia has been reported as being more important than blood pressure and sympathetic nerve activity in evoking apnoea-induced increases in cerebral perfusion (Pan *et al.*, 1997; Przybylowski *et al.*, 2003; Bain *et al.*, 2016). However, the effect of facial cooling on CBF in humans, remains incompletely understood.

To the authors' knowledge, there are only two studies that have investigated the effects of facial cooling on intra-cranial perfusion in healthy humans (Brown *et al.*, 2003; Kjeld *et al.*, 2009). Brown *et al.* (2003) reported a small increase in MCA mean blood flow velocity (MCA V_{mean}) during cold face stimulation (9%), while Kjeld *et al.* (2009) reported that MCA V_{mean} responses to a breath-hold (BH) performed during moderate intensity leg cycling exercise, were augmented when undertaken with concurrent facial immersion. However, the contribution of exercise *per se* to the latter MCA V_{mean} response is unclear. Additionally, these studies did not consider whether thermoreceptor stimulation may have contributed to the responses, and as potential changes in P_aCO_2 were not controlled the powerful effects of P_aCO_2 on CBF regulation secondary to respiratory changes, cannot be excluded. Finally, an important assumptions implicit in the use of MCA V_{mean} as an index of

CBF also limit these investigations. That is, in the absence of MCA diameter measurements, it is assumed that MCA V_{mean} is representative of MCA blood flow.

The purpose of the present study was to determine the contribution of trigeminal nerve stimulation to the diving response associated changes in CBF, and to address the shortcomings mentioned above. To achieve this, cardiovascular and cerebral vascular responses (i.e., MCA V_{mean}) to stimulation of trigeminal afferents with facial cooling (0°C) were determined. Facial cooling trials were undertaken under control conditions (i.e., spontaneous respiration and poikilocapnia) (Protocol 1) and with isocapnia ensured (Protocol 2). Also, the cardiovascular and cerebral vascular responses to another thermoreceptor stimulus, namely the cold pressor test (CPT), were examined (both Protocol 1 and 2). In addition, facial cooling was performed during a breath-hold, in which CO_2 would naturally accumulate and also without a breath-hold (Protocol 3). Finally, breath-hold trials were performed both with and without facial cooling to further elucidate the role of trigeminal nerve stimulation on CBF (Protocol 4). To circumvent issues surrounding the validity of MCA V_{mean} being representative of CBF, internal carotid artery volumetric blood flow was also measured (Protocols 3 and 4). This series of experimental trials permitted us to test the hypothesis that the stimulation of trigeminal afferents with facial cooling contributes to the CBF increases during the diving response.

METHODS

Ethical approval

All study protocols were approved by the Health, Safety and Ethics Committee at the University of Birmingham, School of Sport, Exercise and Rehabilitation Sciences (22/03/16MW and ERN_19-0700), and were undertaken according with the Declaration of Helsinki, except for registration in a database. Written informed consent was acquired from each participant before the commencement of the study following the provision of a detailed verbal and written overview of the experimental procedures.

Participants

A total of 37 volunteers completed this study. Participants were healthy and free of any renal, neurological, cardiovascular, respiratory, or metabolic diseases, and were not using prescribed or over-the-counter medications. Participants were requested to refrain from any caffeinated, or alcoholic beverages and not to undertake vigorous exercise, for 24 hr before the experimental sessions. Participants were not trained breath-hold divers.

Experimental measures

All participants rested in a semi-supine position throughout the study. Heart rate was monitored using an electrocardiography (ECG) and beat-to-beat arterial blood pressure assessed using finger photoplethysmography (Finometer Pro; Finapres Medical Systems, Arnhem, the Netherlands). An automated sphygmomanometer (Tango+; SunTech Medical) was used to verify resting blood pressure measures. A mouthpiece and a nose-clip were worn by participants and partial pressure of end-tidal CO_2 ($P_{ET}CO_2$), used to estimate $PaCO_2$, was sampled at the mouth and was recorded by a calibrated gas analyser (model ML206, ADInstruments, Dunedin, New Zealand). A 2-MHz pulsed Doppler ultrasound probe

(Doppler Box X; Compumedics, Singen, Germany) was used to simultaneously measure the blood flow velocity of the right MCA. The temporal window, approximately 1 cm above the zygomatic arch, was insonated for the MCA (depth 45-65 mm) (Willie *et al.*, 2011). Once the signal was stable, the probe was fixed using a modifiable head kit that locked the angle of insonation at the optimum position allowing signal stability. All measurements recorded were converted from analogue to digital data at 1 kHz (Powerlab, 16/30; ADInstruments) and were stored in for offline analysis (LabChart Pro; ADInstruments).

Duplex Doppler ultrasound (Terason T3300, Teratech, Burlington, MA, USA) was used to measure left internal carotid artery blood flow velocity (ICA_v) and diameter (ICA_d) by a single experimenter (SAS). A 4-15 MHz multi-frequency linear-array transducer was used with a constant insonation of 60° angle relative to the skin. ICA recordings were undertaken at a site 1 to 1.5 cm distal to the carotid bifurcation. For ICA localisation, the brightness mode was used on a longitudinal section to clarify the vessel appearance and assess ICA_d. The pulse-wave mode was used to determine ICA_v. ICA images were captured and stored as video files for offline analysis using automated edge detection software independently of investigator influence (FMD Studio, Pisa, Italy). All video files were analysed by a single operator (SAS).

Experimental protocols

Protocol 1: Facial cooling and CPT under poikilocapnic conditions

Thirteen healthy individuals (11 males, age: 23 [4], height: 174 [7] cm, weight: 74 [8] kg; mean [SD]) undertook trials of facial cooling and CPT in a random order decided with a coin toss. A >15-min rest period was allowed between the trials to allow for the restoration of the measured variables to baseline.

Facial cooling*:* Following a 3-min baseline period, an ice pack (0°C) was used to simulate the trigeminal nerve stimulation component of the diving response for 3 min. The ice pack was shaped so that it covered the areas innervated by the ophthalmic (forehead) and maxillary division (cheeks) of the trigeminal nerve. This was followed by a recovery period of 3 min.

CPT: Following a 3-min baseline period, participants were instructed to immerse their hand up to their wrist into a bucket containing iced-water (4°C) for 3 min. This was followed by removal of the hand and continuation of the data collection for a further 3-min recovery period.

Protocol 2: Facial cooling and CPT under isocapnic conditions

In protocol 2, eight healthy individuals (8 males, age: 23 ± 6, height: 180 ± 7 cm, weight: 74 ± 6 kg) undertook CPT and facial cooling as described for Protocol 1, however $P_{ET}CO_2$ was maintained at baseline values (i.e., $P_{ET}CO_2$ controlled at +1 mmHg baseline) by the manual supplementation of CO_2 to the inspired air.

Protocol 3: Facial cooling with and without breath-hold

Eight healthy individuals completed protocol 3 (7 males, age: 24 ± 3 years, height: 175 ± 4 cm, weight: 72 ± 8 kg). At an initial familiarisation session, participants practiced exhaling and holding their breath for as long as possible on three occasions. At a subsequent experimental session, the cardiovascular and cerebrovascular effects of facial cooling with breath-hold (FC +BH) and without a breath-hold (FC -BH) were determined. The FC +BH trial was always undertaken first because the FC -BH trial was matched in length to the FC +BH trial. For the FC +BH trial, after a 1-min baseline period, participants were instructed to hold their breath at end of a normal expiration and were asked to hold until they reached their

maximum comfortable breath-hold duration (i.e., prior to any straining manoeuvre). At the start of the breath-hold an ice pack (0°C) was placed on the face to simulate the trigeminal nerve stimulation component of the diving reflex for the full length of the breath-hold. Following the release of the breath-hold this protocol was concluded after a 1-min period of recovery. FC +BH and FC -BH trials were separated by a >15-min rest period to allow the restoration of the measured variables to baseline values. The FC -BH trial consisted of a 1-min baseline period followed by facial cooling for the same duration used in the previous trial. Following the completion of facial cooling, this protocol was concluded after a 1-min period of recovery.

Protocol 4: Breath-hold with and without facial cooling

In protocol 4, eight healthy individuals (8 males, age: 24 ± 3 years, height: 175 ± 4 cm, weight: 72 ± 8 kg) undertook BH both with and without facial cooling.

In protocol 4, eight healthy individuals (8 males, age: 24 ± 3 years, height: 175 ± 4 cm, weight: 72 ± 8 kg) undertook breath-hold both with and without facial cooling.

At an initial familiarisation session, participants practiced exhaling and holding their breath for as long as possible on three occasions. At a subsequent experimental session, the cardiovascular and cerebrovascular effects of breath-hold with (BH +FC) and without facial cooling (BH -FC) were determined. Trials were randomised with the order decided by a coin toss. Trials were matched in length with the duration of the second trial being matched to the first trial. Each trial was preceded by a 1-min baseline period, then participants were asked to hold their breath at end of a normal expiration, and to hold this until they reached their maximum comfortable breath-hold duration (i.e., prior to any straining manoeuvre) (first trial) or until requested to return to normal breathing (second trial). Following the release of the breath-hold a 1-min recovery period was conducted. For the BH +FC trial only, at the

start of the breath-hold an ice pack (0°C) was placed on the face to simulate the facial cooling component of the diving reflex for the full length of the breath-hold. A recovery period (>15-min) was allowed between the trials to allow for the restoration of the measured variables to baseline.

Data analysis

MAP was calculated as Razminia *et al.* (2004):

$$MAP = \left(\frac{Systolic\ BP - Diastolic\ BP}{3}\right) + Diastolic\ BP$$

Volumetric blood flow was (Flück *et al.*, 2017):

$$ICA_Q = \mathrm{ICA}_v \cdot [\pi\ (0.5\ \cdot ICA_d)^2]\ x\ 60$$

Cerebrovascular conductance index (CVCi) was (Flück *et al.*, 2017):

$$\mathrm{MCA\ CVCi}\ \frac{\mathrm{MCA}v_{mean}}{\mathrm{MAP}}$$

$$\mathrm{ICA\ CVC}\ = \frac{ICA_Q}{\mathrm{MAP}}$$

Statistical analysis

Statistical analysis was performed using SigmaPlot (version 13.0, SYSTAT Software Inc., Chicago, IL, USA). Physiological data were statistically analysed using repeated-measures analysis of variance (ANOVA) with significant main effects and interactions examined *post hoc* using Student Newman-Kuels tests. More specifically, to determine the physiological responses to facial cooling and CPT under poikilocapnic conditions (Protocol 1) averages were calculated for baseline (3 min), facial cooling and CPT interventions on a minute-by-minute basis, and recovery (3 min). A two-way repeated-measures ANOVA was

used in which the factors were condition (FC, CPT) and time (baseline, intervention min 1-3, recovery), as well as the interaction between them. Given the known association between changes in $P_{ET}CO_2$ and MCA V_{mean}, Pearson correlations were used to examine the change from baseline in MCA V_{mean} and $P_{ET}CO_2$ during the 3rd minute of both facial cooling and CPT. To better understand the $P_{ET}CO_2$-independent influence of facial cooling and CPT on the cerebrovascular response, Protocol 2 was used to determine the physiological responses to facial cooling and CPT under isocapnic conditions. Averages were calculated over the same time points, and the same ANOVA approach used, as described for Protocol 1. In Protocol 3, facial cooling was examined both with (+BH) and without (-BH) a breath-hold, because during diving a breath-hold accompanies facial cooling. ICA_Q was also measured along with MCA V_{mean}, thus variables were averaged at baseline (1 min), the last 10 cardiac cycles of either facial cooling with (FC +BH) or without (FC -BH) a breath hold, and during recovery (1 min). A two-way repeated-measures ANOVA was used in which the factors were condition (FC +BH, FC -BH) and time (baseline, facial cooling, recovery), as well as the interaction between them. In Protocol 4, physiological responses to a breath-hold (BH) were examined when undertaken with (+FC) and without (-FC) facial cooling. A two-way repeated-measures ANOVA was used in which the factors were condition (BH +FC, BH -FC) and time (baseline, BH, recovery), as well as the interaction between them. To compare responses across protocols, a 1-way ANOVA was used to compare the change in MCA V_{mean} from baseline for the pokilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), facial cooling without a breath-hold (FC -BH; Protocol 3), facial cooling with a breath-hold (FC +BH; Protocol 3 and Protocol 4), and a breath-hold alone (BH –FC) trials. Data are displayed as mean ± SD, unless otherwise indicated. Differences were considered significant when $P<0.05$.

RESULTS

Protocol 1: Facial cooling and CPT under poikilocapnic conditions

Cardiovascular and cerebrovascular responses to facial cooling and CPT under poikilocapnic conditions are shown in Table 1. During facial cooling, MCA V_{mean}, MAP, MCA CVCi and $P_{ET}CO_2$ remained unchanged, while HR was numerically reduced (P=0.13 baseline vs. min 3). During CPT, MCA V_{mean} remained unchanged, while MAP (P<0.05 vs. baseline at min 2-3, P<0.01) and HR were increased (P<0.05 vs. facial cooling), and MCA CVCi (P<0.05 baseline vs. min 2-3) and $P_{ET}CO_2$ were reduced (P<0.05 baseline vs. min 2-3).

Given that reductions in $P_{ET}CO_2$ are well known to result in cerebral vasoconstriction, the association between changes in $P_{ET}CO_2$ and MCA V_{mean} and during the facial cooling and CPT conditions was examined. A moderate positive correlation was observed between the change from baseline in $P_{ET}CO_2$ and MCA V_{mean} measured during the last minute of both facial cooling (r=0.59: P=0.04) and CPT (r=0.64: P=0.03).

Protocol 2: Facial cooling and CPT under isocapnic conditions

Given the observation made in Protocol 1 that facial cooling and CPT mediated changes in $P_{ET}CO_2$ are significantly associated with response in MCA V_{mean}, trials of facial cooling and CPT were repeated in Protocol 2 under isocapnic conditions. The aim being to unmask the $P_{ET}CO_2$-independent influence of facial cooling and CPT on the cerebrovascular response. Accordingly, the cardiovascular and cerebrovascular responses to facial cooling and CPT performed under isocapnic conditions are shown in Table 2.

During isocapnic facial cooling, MCA V_{mean} was unchanged (P>0.05), MAP was increased (P<0.05 baseline vs. min 2 and 3), while HR (P<0.05 baseline vs. min 2) and MCA CVCi (P<0.05 baseline vs. min 2 and 3, respectively) decreased. During isocapnic CPT, MCA V_{mean} (P<0.05 baseline vs. min 1 and 2), MAP (P<0.05 baseline vs. min 1 and 2), and

HR ($P<0.05$ CPT vs. facial cooling) were increased, while MCA CVCi was reduced ($P<0.05$ baseline vs. min 2 and 3).

Protocol 3: Facial cooling with and without breath-hold

To better discern the cerebrovascular consequences of facial cooling, ICA_Q was measured along with MCA V_{mean} in Protocol 3. In addition, because during diving a breath-hold accompanies facial cooling, in Protocol 3 facial cooling was examined both with (FC +BH) and without (FC -BH) a breath-hold. The apnoea was held for 26 ± 4 s. MCA V_{mean} and ICA_Q were only increased when facial cooling was accompanied by a breath-hold ($P<0.05$ vs. baseline), while ICA_Q and ICA_v were different between trials ($P<0.05$ FC +BH vs FC -BH) (Table 3). MAP was elevated numerically during FC -BH trial ($P=0.23$ vs baseline), while MAP increased during the FC +BH trial ($P<0.05$ vs. FC -BH). HR was unchanged during the FC –BH trial ($P>0.05$ vs. baseline) but declined in the FC +BH trial ($P=0.01$ vs. baseline). MCA CVCi and ICA CVC remained unchanged in both trials ($P>0.05$ vs. baseline).

Protocol 4: Breath-hold with and without facial cooling

To further understand the cerebrovascular effects of facial cooling, in Protocol 4 the responses to a breath-hold were determined both with and without facial cooling (Table 4). The apnoea was held for 28 ± 4 s. A breath-hold undertaken either with facial cooling (+FC) or without facial cooling (-FC) increased MCA V_{mean}, ICA_Q, MAP, MCA CVCi, and ICA_v from baseline ($P<0.05$) with no difference between conditions.

Comparison of MCA V_{mean} responses in Protocols 1-4

As illustrated in Figure 1, MCA V_{mean} responses to poikilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), and facial cooling without a breath-hold (FC -BH; Protocol 3) were minimal, and lower than that evoked by facial cooling when accompanied by a breath-hold (FC +BH, Protocol 3; BH +FC, Protocol 4), and a breath-hold undertaken in the absence of facial cooling (BH -FC, Protocol 4) ($P<0.05$).

DISCUSSION

We sought to determine the contribution of facial cooling (i.e., trigeminal nerve stimulation) to changes in CBF during the diving response. In order to examine the influence of potentially modulatory factors associated with diving (e.g. apnoea, changes in $P_{ET}CO_2$, thermoreceptor stimulation), we implemented different protocols to isolate these variables The major novel findings are that in young healthy individuals, 1) MCA V_{mean} did not increase during facial cooling or CPT under poikilocapnic conditions (Protocol 1), 2) under isocapnic conditions MCA V_{mean} did increase during thermoreceptor stimulation with CPT, but not during CPT (Protocol 2), 3) both MCA V_{mean} and ICA_Q were increased when facial cooling was combined with a breath-hold, but not when facial cooling was performed with spontaneous breathing (Protocol 3), and 4) similar increases in MCA V_{mean} and ICA_Q were observed during a breath-hold when performed either alone or in combination with facial cooling (Protocol 4). Collectively, our findings suggest that physiological factors associated with breath holding (e.g., pressor response, CO_2 accumulation) make the predominant contribution to diving response mediated-increases in CBF in humans.

Cerebral perfusion during facial cooling

During diving, a multitude of mechanisms can contribute to the regulation of CBF, including neurogenic and hemodynamic factors, neurovascular coupling, and changes in blood gases (Bain *et al.*, 2018). The findings of Ollenberger *et al.* (1998) in rats indicate that trigeminal nerve stimulation can play a role in regulation of CBF. They observed a redistribution of blood flow away from the periphery to the brain during swimming, but only if the head was submerged (i.e., with trigeminal nerve stimulation / facial cooling) and not when the head remained above water. As reviewed by Lapi *et al.* (2016), stimulation of the trigeminal cardiac reflex, involving sensory ending of the trigeminal nerve, evokes a (partly)

nitric oxide-mediated cerebrovascular vasodilatation in rabbits. Interestingly, the direct stimulation of the trigeminal root has been reported not to cause dilatation of the pial arteries in cats and monkeys, whereas stimulation of either the facial nerve root or the vagus nerve does evoke cerebral vasodilatation (Cobb & Finesinger, 1932). How trigeminal nerve stimulation can regulate cerebral flow in humans remains less well studied. Brown *et al.* (2003) reported that trigeminal afferent activation increases MCA V_{mean} (by 9%) when evoked with the cold face test under poikilocapnic conditions in healthy individuals. In contrast, in our study we found no increases in MCA V_{mean} during facial cooling under poikilocapnic conditions. A potential explanation for these contradictory findings may be differences in $P_{ET}CO_2$, well recognised as a powerful dilator of the cerebral vasculature. In the present study we observed a moderate positive relationship between $P_{ET}CO_2$ and MCA V_{mean} ($r=0.59$; $p=0.04$) during facial cooling (Protocol 1), and although overall under poikilocapnic conditions no differences from baseline in $P_{ET}CO_2$ were noted, a significant degree of between-subject variability was observed (i.e., responses ranged from +3.5 to -5.5 mmHg) likely a result of a heterogeneous ventilatory response. To further examine the influence of $P_{ET}CO_2$ on cerebral perfusion during facial cooling, we repeated the facial cooling under isocapnic conditions (Protocol 2). However, even under consistent isocapnic conditions no changes in MCA V_{mean} were observed during trigeminal nerve stimulation by facial cooling.

Another possible explanation for previous reports of an increase in CBF during the cold face test is activation of thermoreceptors. Signals from cutaneous thermoreceptor afferents are integrated within the central nervous system (e.g., within hypothalamic and medullary regions) and lead to activation of cortical sites (Di Piero *et al.*, 1994), which may increase local perfusion by neurovascular coupling. Under poikilocapnic conditions MCA V_{mean} remained unchanged (Protocol 1), likely as a result of a hyperventilation induced fall in

$P_{ET}CO_2$ decreased secondary to hyperventilation. Whereas, under isocapnic conditions (i.e., $P_{ET}CO_2$ controlled at +1 mmHg baseline) MCA V_{mean} increased during CPT (Protocol 2). Such findings agree with those of Tymko *et al.* (2017) and highlight the importance of nociceptor mediated alterations in ventilation and thus $P_{ET}CO_2$, on blunting the cerebral perfusion response to the CPT. A striking example of the balance between the effects of ventilation (and thus $P_{ET}CO_2$) on cerebral perfusion in the cold has been provided by Datta and Tipton (2006). They reported that reductions in MCA V_{mean} observed in hyperventilating participants immersed up to the neck in cold water (12°C) were less marked than when reductions in $P_{ET}CO_2$ were matched in control experiments undertaken in either thermoneutral water (35°C) or room air (24°C). Such findings suggest that under conditions of more extreme cold stress, the vasoconstrictor effects of hyperventilation on the cerebral vessels may at least be partially offset by other factors, such as neurovascular coupling and MAP.

CBF and apnoea

Several studies show that an apnoea robustly increases cerebral perfusion (Pan *et al.*, 1997; Przybylowski *et al.*, 2003; Kjeld *et al.*, 2009; Bain *et al.*, 2016). For example, Przybylowski *et al.* (2003) reported dramatic increases in MCA V_{mean} (by 42 %) during a short 20 s apnoea, while Kjeld *et al.* (2009) have shown that MCA V_{mean} increased from a baseline of 37 ± 23 cm s^{-1} to 103 ± 15 cm s^{-1} during a maximal apnoea. In the present study we observed that when facial cooling was undertaken in combination with an apnoea, MCA V_{mean} increased by 23 % (Protocol 3). In addition, we observed that ICA_Q also increased during facial cooling with a concurrent apnoea (by 26 %) (Protocol 3). In fact, MCA V_{mean} and ICA_Q only increased when facial cooling was accompanied by an apnoea and did not increase during a cold face stimulation with uncontrolled breathing (poikilocapnic

conditions). Moreover, when an apnoea was performed either alone or in combination with facial cooling similar increases in MCA V_{mean} and ICA_Q were observed (Protocol 4). Such findings suggest that physiological factors associated with breath holding make the predominant contribution to diving response mediated-increases in CBF in humans.

The CBF responses to an apnoea may be attributed to a number of factors, which include metabolic, neurogenic and hemodynamic factors, neurovascular coupling, and changes in blood gases (Bain, *et al.* 2018). Increases in MAP were noted during breath-holding and these may be partially responsible for the increase in cerebral perfusion during apnoea. Indeed, Przybylowski *et al.* (2003) demonstrated that ganglionic blockade with trimethaphan eliminated the increase in MAP during a 20 s apnoea, and the MCA V_{mean} response was significantly blunted (62% of hyperaemic response without ganglionic blockade). Thus, in addition to increases in P_aCO_2 alteration in MAP likely makes a contribution to the increase in cerebral perfusion noted during apnoea.

Methodological considerations

The findings of the present study should be considered in light of the following:

1) Study population: Care should be taken when generalising the findings of the current study to a population beyond the young healthy group studied. The sympathetic and blood pressure responses to facial cooling are reportedly modified in some disease states (Prodel *et al.*, 2017) and therefore it is quite likely that the cerebrovascular responses are altered too. In rat models of traumatic brain injury, trigeminal nerve stimulation was reported to increase CBF and reduce the development of secondary injury symptoms, such as oedema, blood-brain barrier disruption, and lesion volumes (Chiluwal *et al.*, 2017). In humans, therapeutic use of trigeminal nerve stimulation using external electrical stimulation has been examined in neurologic, cardiovascular and psychiatric conditions such as, epilepsy,

depression, attention deficit hyperactivity disorder and post-traumatic stress disorder (Grahame & Hann, 1978; Cook *et al.*, 2015; Borsody & Sacristan, 2016; Cook *et al.*, 2016). This approach resulted in reduced CBF in regions attributed with initiation and propagation of seizures, whereas CBF was enhanced in other cortex regions where metabolism is low because of depression (Cook *et al.*, 2016). In addition, elegant work by Schaller (2005) has documented that stimulation of the trigeminal nerve during craniofacial surgery in anaesthetised patients can evoke a trigemino-cardiac reflex, with potential implications for CBF (Schaller, 2004). Comparisons of these clinical studies to the present work are difficult due to differences in the mode of trigeminal afferent activation and the presence of pathology. We acknowledge that it would have been ideal for all participants to take part in each experimental session, however due to logistical reasons this was not possible. Finally, we acknowledge that the majority of participants in the present study were men and we have not been able to include a comparison of sex-differences in the present analysis. Whether there are sex-differences in the CBF responses to facial cooling requires further study.

2) $P_{ET}CO_2$: P_aCO_2 was not directly measured and instead was indexed using $P_{ET}CO_2$. Young *et al.* (1991) identified similar hypercapnic cerebrovascular reactivity when either P_aCO_2 or the surrogate $P_{ET}CO_2$ was used. However, this relationship was only consistent while participants maintained a fixed supine position. Therefore, in our study subjects remained in a comfortable supine position throughout the data collection period.

3) Assessment of CBF: Cerebral perfusion was principally assessed using transcranial Doppler ultrasound measures of MCA V_{mean}, which, in the absence of a direct measurement of MCA diameter, can only be assumed to reflect MCA blood flow. However, studies were also included where ICA measures of blood flow were derived from simultaneous duplex Doppler ultrasound measurements of ICA diameter and velocity (Protocols 3 and 4, but not Protocols 1 and 2). Of note, the facial cooling and facial cooling with concomitant apnoea

evoked very similar responses in ICA_Q to that exhibited in MCA V_{mean}. However, it remains to be determined whether the MCA V_{mean} and ICA_Q responses described are representative of perfusion changes in other major cerebral arteries (e.g., vertebral and posterior cerebral arteries), which given the known regional differences in cerebral vascular regulation may not be the case.

Summary

The findings of the present study indicate that factors associated with breath-holding (e.g., arterial CO_2 accumulation, pressor response), rather than stimulation of cutaneous trigeminal afferents, makes the predominate contribution to diving response mediated increases in CBF in humans.

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ADDITIONAL INFORMATION

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COMPETING INTERESTS

The authors have no conflicting interests to declare.

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AUTHOR CONTRIBUTIONS

S.E.A., I.D.B and J.P.F. conceived and designed research; I.D.B., S.E.A., A.A and R.T.J. performed experiments; S.E.A. analysed data; S.E.A. and J.P.F. interpreted results of experiments; S.E.A., and J.P.F prepared figures; S.E.A. and J.P.F drafted manuscript; R.T.J., S.E.A., A.A and J.P.F. edited and revised manuscript. All authors approved final version of manuscript.

Table 1. Cardiovascular and cerebrovascular responses to facial cooling (FC) and cold pressor test (CPT) under poikilocapnic conditions (Protocol 1).

		Baseline	Intervention (min)			Recovery	P-Value		
			1	2	3		Condition	Time	Int.
MCA V_{mean} (cm s^{-1})	FC	52 ± 7	53 ± 8	52 ± 9	52 ± 8	52 ± 7	0.61	0.59	0.70
	CPT	53 ± 16	55 ± 15	52 ± 13	52 ± 13	53 ± 15			
MAP (mmHg)	FC	86 ± 7	86 ± 9	88 ± 8	89 ± 10	86 ± 8	0.16	**<0.01**	**<0.01**
	CPT	85 ± 5	88 ± 11	101 ± 17*†‡	98 ± 10*†‡	87 ± 7			
HR (b min^{-1})	FC	68 ± 12	66 ± 11	66 ± 12	64 ± 12	69 ± 12	**0.03**	0.21	**<0.01**
	CPT	69 ± 10	73 ± 9*†	71 ± 9†	69 ± 11†‡	68 ± 11			
MCA CVCi (cm s^{-1}/mmHg)	FC	0.60 ± 0.14	0.60 ± 0.13	0.59 ± 0.12	0.58 ± 0.11	0.61 ± 0.13	0.63	**<0.01**	**<0.01**
	CPT	0.63 ± 0.18	0.62 ± 0.10	0.52 ± 0.10*†‡	0.54 ± 0.12*‡	0.61 ± 0.17			
$P_{ET}CO_2$ (mmHg)	FC	39 ± 4	40 ± 4	40 ± 5	40 ± 5	39 ± 4	**<0.01**	0.08	**<0.01**
	CPT	40 ± 4	39 ± 4†	37 ± 5*†‡	38 ± 4*†‡	39 ± 3			

MCA V_{mean}, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * P<0.05 vs. Baseline, † P<0.05 vs. FC, ‡ P<0.05 vs. Min 1.

Table 2. Cardiovascular and cerebrovascular responses to facial cooling (FC) and cold pressor test (CPT) under isocapnic conditions (Protocol 2).

		Baseline	Intervention (min)			Recovery	P-Value		
			1	2	3		Condition	Time	Int.
MCA V_{mean} (cm s^{-1})	FC	57 ± 12	55 ± 13	54 ± 14	56 ± 15	56 ± 12	0.06	0.10	**<0.01**
	CPT	58 ± 8	63 ± 13*†	65 ± 11*†	65 ± 13*†	60 ± 10			
MAP (mmHg)	FC	87 ± 6	91 ± 12	102 ± 8*†‡	100 ± 8*†‡	89 ± 5	**0.00**	**<0.01**	**0.02**
	CPT	90 ± 6	97 ± 13*†	112 ± 13*†‡	111 ± 10*†‡	96 ± 7			
HR (b min^{-1})	FC	65 ± 10	65 ± 11	58 ± 9*‡	60 ± 11	64 ± 12	**0.04**	**<0.01**	**<0.01**
	CPT	67 ± 11	77 ± 14*†	70 ± 12†	66 ± 9	61 ± 6†			
MCA CVCi (cm s^{-1}/mmHg)	FC	0.80 ± 0.23	0.79 ± 0.30	0.64 ± 0.19	0.67 ± 0.21	0.77 ± 0.21	0.58	**<0.01**	0.54
	CPT	0.75 ± 0.14	0.76 ± 0.21	0.66 ± 0.12*	0.66 ± 0.12*	0.71 ± 0.12			
$P_{ET}CO_2$ (mmHg)	FC	41 ± 5	41 ± 5	40 ± 5	41 ± 5	41 ± 5	0.89	0.37	0.37
	CPT	41 ± 4	42 ± 5	41 ± 5	40 ± 6	41 ± 4			

MCA V_{mean}, middle cerebral artery mean flow velocity; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; $P_{ET}CO_2$, partial pressure of end-tidal CO_2; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * P<0.05 versus Baseline, † P<0.05 vs. FC, ‡ P<0.05 vs. Min 1.

628 **Table 3.** Cardiovascular and cerebrovascular responses to facial cooling (FC) undertaken without (-BH) and with (+BH) a breath-hold (Protocol
629 3).

		Baseline	FC	Recovery	P-Value		
					Condition	Time	Int.
MCA V_{mean} (cm s^{-1})	-BH	66 ± 21	66 ± 21	66 ± 23	0.51	**<0.01**	**<0.01**
	+BH	67 ± 26	82 ± 24*	66 ± 25			
ICA_Q (ml min^{-1})	-BH	182 ± 68	177 ± 70	181 ± 74	0.12	**<0.01**	**<0.01**
	+BH	185 ± 72	232 ± 95*†	180 ± 70			
MAP (mmHg)	-BH	87 ± 5	91 ± 6	88 ± 6	0.21	**<0.01**	**<0.01**
	+BH	88 ± 5	101 ± 11*†	86 ± 5			
HR (b min^{-1})	-BH	67 ± 10	66 ± 10	66 ± 9	0.76	0.07	0.13
	+BH	72 ± 5	65 ± 11	66 ± 5			
MCA CVCi (cm s^{-1}/mmHg)	-BH	0.70 ± 0.23	0.69 ± 0.22	0.69 ± 0.26	0.71	0.78	0.08
	+BH	0.77 ± 0.30	0.81 ± 0.22	0.77 ± 0.31			
ICA_v (cm s^{-1})	-BH	34 ± 10	32 ± 11	33 ± 12	0.37	0.06	**<0.01**
	+BH	35 ± 10	41 ± 14*†	34 ± 10			
ICA_d (cm)	-BH	0.50 ± 0.08	0.50 ± 0.07	0.50 ± 0.08	0.46	0.16	0.20
	+BH	0.47 ± 0.07	0.48 ± 0.08	0.47 ± 0.08			

ICA CVC ($ml\ min^{-1}/mmHg$)	-BH	2.1 ± 0.8	2.3 ± 0.8	2.1 ± 0.9	0.35	0.87	0.12
	+BH	2.1 ± 0.8	2.0 ± 1.0	2.1 ± 0.8			

MCA V_{mean}, middle cerebral artery mean flow velocity; ICA_Q, internal carotid artery flow; MAP, mean arterial pressure; HR, heart rate; CVCi, cerebrovascular conductance; ICA_v, internal carotid artery velocity; -BH, facial cooling without breath-hold; +BH, facial cooling with breath-hold; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * $P<0.05$ vs. Baseline, † $P<0.05$ vs. -BH.

635 **Table 4.** Cardiovascular and cerebrovascular responses to breath-hold undertaken without (-FC) and with (+FC) facial cooling (Protocol 4).

		Baseline	Breath-hold	Recovery	P-Value		
					Condition	Time	Int.
MCA V_{mean} (cm s^{-1})	-FC	47 ± 7	59 ± 11	47 ± 8	0.62	**<0.01**	0.94
	+FC	46 ± 7	57 ± 14	47 ± 8			
ICA_Q (ml min^{-1})	-FC	190 ± 107	236 ± 150	203 ± 139	0.48	**<0.01**	0.29
	+FC	206 ± 60	227 ± 89	187 ± 70			
MAP (mmHg)	-FC	90 ± 8	96 ± 9	90 ± 9	0.36	**0.03**	0.37
	+FC	90 ± 9	103 ± 20	98 ± 20			
HR (b min^{-1})	-FC	73 ± 14	69 ± 16	72 ± 14	0.20	**<0.01**	0.36
	+FC	76 ± 13	71 ± 16	73 ± 14			
MCA CVCi (cm s^{-1}/mmHg)	-FC	0.53 ± 0.10	0.62 ± 0.14	0.53 ± 0.10	0.48	**<0.01**	0.53
	+FC	0.52 ± 0.10	0.59 ± 0.19	0.50 ± 0.15			
ICA_v (cm s^{-1})	-FC	24 ± 9	28 ± 11	24 ± 7	0.48	**0.01**	0.53
	+FC	28 ± 6	31 ± 11	26 ± 8			
ICA_d (cm)	-FC	0.53 ± 0.08	0.52 ± 0.08	0.52 ± 0.09	0.63	0.71	0.61
	+FC	0.53 ± 0.08	0.53 ± 0.09	0.52 ± 0.08			
ICA CVC (ml min^{-1}/mmHg)	-FC	2.2 ± 1.2	2.6 ± 1.8	2.3 ± 1.6	0.83	0.11	0.42

+FC	2.3 ± 0.8	2.3 ± 1.1	2.1 ± 0.9

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637 MCA V_{mean}, middle cerebral artery mean flow velocity; ICA_Q, internal carotid artery flow; MAP, mean arterial pressure; HR, heart rate; CVCi,
638 cerebrovascular conductance; ICA_v, internal carotid artery velocity; -FC, breath-hold without facial cooling; +FC, breath-hold with facial
639 cooling; Int, interaction. Values are mean ± SD. P values represent two-way repeated ANOVA results. * $P<0.05$ versus Baseline.

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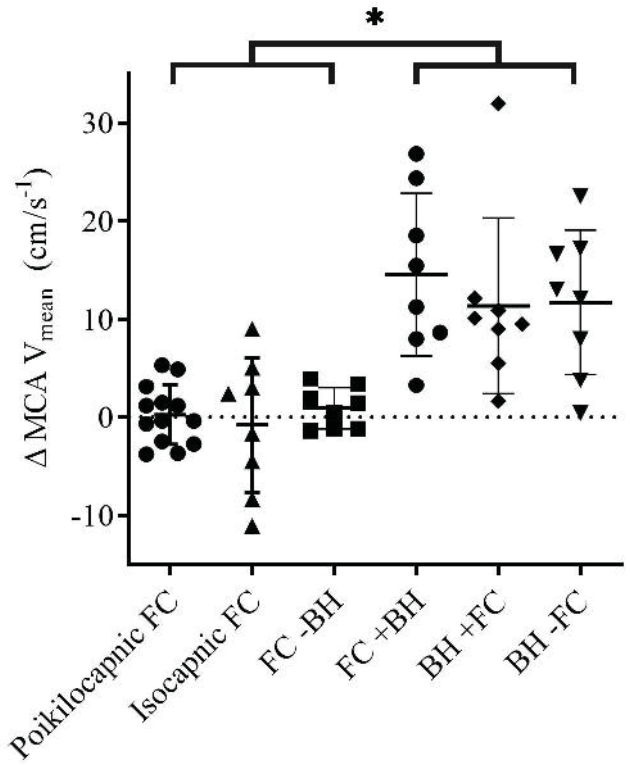
FIGURE LEGEND

Figure 1. Comparison of the MCA V_{mean} responses evoked during combinations of facial cooling (FC) and breath-hold (BH). MCA V_{mean} responses to poikilocapnic facial cooling (Protocol 1), isocapnic facial cooling (Protocol 2), and facial cooling without a breath-hold (FC -BH; Protocol 3) were minimal, and significantly attenuated in comparison to facial cooling with a breath-hold (FC +BH, Protocol 3; BH +FC, Protocol 4), and a breath-hold undertaken in the absence of facial cooling (BH –FC, Protocol 4). * $P<0.05$, FC +BH, BH +FC, BH –FC conditions were all significantly different from Poikilocapnic FC, Isocapnic FC and FC –BH conditions. Horizontal bars show mean and SD.


*
30
20
10
0
-10
Δ MCA V mean (cm/s-1)
Poikilocapnic FC
Isocapnic FC
FC -BH
FC +BH
BH +FC
BH -FC